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Direct microbial production of prebiotic and antioxidant chitin-oligosaccharides from shrimp byproducts

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ABSTRACT

The chitinous content of crustacean byproducts is an attractive proposition for the production of chitin-oligosaccharides that can be exploited in various biotechnological applications. The currently applied methods for the preparation of chitin-oligosaccharides depend mainly on either chemical or enzymatic hydrolysis of chitin. These methods are suffering from some drawbacks that confined its industrial application. In the current study, the production of chitin-oligosaccharides was performed by the direct bacterial hydrolysis of shrimp byproducts using *Bacillus cereus* strain SSW1. A sequential optimization of the hydrolysis process was achieved by applying Plackett-Burman design followed by central composite design. The optimum chitin-oligosaccharide level of 16.4mg/g was achieved under the optimized fermentation conditions in which 5g of microwave pretreated shrimp byproducts moistened with 10mL of moistening agent composed of K_2HPO_4, 1.5%; $MgSO_4$, 0.01%; KCl, 0.1%, and $FeSO_4.7H_2O$, 0.01% was incubated for 4days at 37°C. After the additional autoclaving process, the amount of the reducing sugars released in the fermentation broth was increased by 32% to reach 21.7mg/g. The resulted product was analyzed by thin-layer chromatography and Fourier Transform Infrared Spectroscopy, confirming the release of a mixture of chitin-oligosaccharide. Finally, the in vitro prebiotic and antioxidant activity of the purified oligosaccharides was determined.

INTRODUCTION

Chitin is one of the most abundant structural biopolymer in nature. It consists of N-acetyl-D-glucosamine monomers linked to each other in a linear form by β-1,4-glycosidic linkages. Among all natural resources of chitin, the highest content exists in crustacean byproducts especially shrimp and crab shells (**Kandra *et al*., 2012**).

The chitin rich byproducts in the aquatic medium can be degraded by the effect of naturally existent chitinolytic bacteria. Consequently, even so there are more than 10^{11} tons of these materials produced every year in the aquatic medium, they do not accumulate in the ocean sediments indicating that the natural chitinolytic machineries are sufficiently efficient to handle this enormous byproducts (**Ghorbel-Bellaaj *et al*., 2012**). On the other hand, the human consumption of crustaceans results in the accumulation of

numerous inedible parts that discarded by incineration, ocean dumping and land filling. Recently, shell biorefinery was created for the production of various valuable compounds (**Yadav *et al.*, 2019**). From these compounds, chitin and its hydrolysis products (oligosaccharides and N-acetyl-D-glucosamine) have attracted a growing interest due to their various health benefits (**Liaqat and Eltem, 2018 and Das *et al.*, 2019**). Chitin-oligosaccharides (COS) can help to maintain a balanced gut ecosystem and consequently decrease the risk of intestinal diseases (**Selenius *et al.*, 2018 and Zheng *et al.*, 2018**) in addition to their various biological activities as antibacterial (**Benhabiles *et al.*, 2012**), antioxidant (**Halder *et al.*, 2013**) and anticancer (**Salah *et al.*, 2013**).

Reportedly, the hydrolysis of chitin could be performed by different chemical and/or enzymatic processes. These processes can help in the reduction of the molecular weight of chitin and release of small fractions with various amino content and degree of acetylation as well as various degree of polymerization (DP) (**Ismail *et al.*, 2019a**). However, the several manipulation steps, environmental hazards and the high cost of the used enzymes are considered serious drawbacks.

COS are either homo or hetero chains of N-acetyl-D-glucosamine and/or D-glucosamine linked to each other by β-1,4-glycosidic linkage. The DP is ranged from 2 to 20 units and it has been found that oligomers with high DP possessed more biological activities than those with low DP. Additionally, they have been proved to achieve various applications in different fields of agricultural, pharmaceutical and food industry (**Liaqat and Eltem, 2018**).

Despite a great interest is focused on optimizing the microbial production of chitinolytic enzymes using either chitin or chitinious byproducts (**Shivalee *et al.*, 2018 and Ismail *et al.*, 2019b**) as well as the application of chitinolytic enzymes in the hydrolysis of chitin and production of COS (**Ismail *et al.*, 2019a and Hou *et al.*, 2020**), the exploitation of chitinous byproducts for the direct microbial production of COS is still rather rare. Apart of this, the industrial production of biological products requires the optimization of the production process in order to achieve the maximum production and consequently decrease the cost. Response surface methodology (RSM) is an efficient statistical tool that has been widely applied in the optimization of various microbiological and biotechnological processes (**Yolmeh & Jafari, 2017 and Hashem *et al.*, 2018**).

The present study concerned with the statistical optimization of the direct produced COS result from the fermentation of shrimp byproducts using *Bacillus cereus* strain SSW1. The factors that influence the production process were screened and standardized by the sequential application of Plackett-Burman design (PBD) and central composite design (CCD) of RSM. Finally, the prebiotic and the antioxidant activity of the purified COS were examined.

MATERIALS AND METHODS

1. Materials

Chitin and N-acetyl glucosamine were purchased from Sigma-Aldrich, Saint Louis, USA. N-acetyl chitopentose and N-acetyl chitohexose were purchased from Seilkagaku Biobusiness Corporation, Tokyo, Japan. Dinitrosalicylic acid (DNS) was obtained from Panreac, Barcelona, Spain. Silica gel 60 thin-layer chromatography (TLC) plates were purchased from Merck, Darmstadt, Germany. All other chemicals were of analytical or HPLC grade.

2. Shrimp byproducts

To prepare the shrimp processed byproducts (SPB); the cephalothoraxes and carapaces parts of marine shrimp were collected from the local seafood market and washed several times with warm tap water to remove impurities as well as soluble organics. The washed SPB parts were boiled for 1h in water and air dried then ground by a standard grinder to fine powder for further use (**Benhabiles *et al*., 2012**).

3. Microorganism and culture conditions

In the current study, the bacterial strain *Bacillus cereus* SSW1 (accession number MK533796) was initially maintained on nutrient agar slants for 24h at 37°C. The cultured bacteria was used to cultivate 5g of SPB moistened with 10mL of moistening agent composed of K_2HPO_4, 0.15%; $MgSO_4$, 0.01%; KCl, 0.2%; and $FeSO_4.7H_2O$, 0.01% then incubated for 24h at 30°C. The concentration of the salts was selected on the base of previous studies concerned with the microbial production of chitin and chitosan degrading enzymes (**Hashem *et al*., 2018 and Ismail *et al*., 2019b**). At the end of the fermentation period, the fermented substrate was extracted with 50mL of distilled water at 150rpm for 1h, boiled for 10min to denaturate the produced enzymes then centrifuged at 5000rpm (4°C) for 10min.

4. Estimation of oligosaccharide level

The level of the total released COS was determined in term of the amount of the released reducing sugars as reported by **Embaby *et al*., (2018)**. In the current study, the total amount of the released reducing sugars was determined according to **Miller, (1959)** using N-acetyl glucosamine as a standard. Briefly, 2.5mL of DNS was added to 1mL of the suitably diluted cell free supernatant then the developed color after boiling for 10min was measured at 540nm. The hydrolysis percentage was calculated as follow:

Hydrolysis percentage = [Total reducing sugars content in the cell free supernatant (mg/g dry SPB)/Total carbohydrate content of complete acid hydrolyzed SPB (mg/g dry SPB)] x 100 Eq. (1)

The total carbohydrate content was determined for SPB according to **Dubios *et al*., (1956)** after its complete acid hydrolysis as described by **Fisher and Dörfel, (1955)**.

5. Optimization of oligosaccharide production

The process of production of COS during the fermentation process of SPB was optimized by two phase model. Initially, the identification of the variables that have the highest influence on the productivity was manifested by applying PBD then in the second phase CCD was applied.

5.1. Plackett-Burman design

Seven independent variables including the fermentation period, the temperature, the period of microwave pretreatment of SPB, the concentration of K_2HPO_4, $MgSO_4$, KCl and $FeSO_4.7H_2O$ were investigated by applying PBD (**Plackett and Burman, 1946**) in eight experimental runs. Each variable is represented in terms of high (+1) and low (-1) values (Table 1). Each generated response was calculated according to the first order linear equation:

$$Y= B_0 +\Sigma B_i X_i \quad \text{Eq. (2)}$$

where Y is the response (released reducing sugars), B_0 is the model intercept and B_i is the linear coefficient and X_i is the level of the independent variable.

The main effect of each variable was determined by the following equation:

$$E_{(Xi)} = 2(\Sigma M_{i+} - M_{i-})/N \quad \text{Eq. (3)}$$

where $E_{(Xi)}$ is the effect of the tested variable. M_{i+} and M_{i-} represent the released reducing sugars from the experimental runs where the independent variable (Xi) was present at high and low values respectively and N is the number of runs.

5.2. Central composite design

In the current study, the highest significant independent two variables were examined by applying a CCD (**Box and Wilson, 1951**) with four-star points and five-replicates at the center point in 13 experimental runs. The second order polynomial function to correlate relationship between the independent variables and the response of the released reducing sugars was as follow:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_1\beta_2 X_1 X_2 \quad \text{Eq. (4)}$$

where Y is the released reducing sugars (mg/mL), β_0 is the intercept, β_1 and β_2 are linear coefficients, β_{11} and β_{22} are quadratic coefficients, β_{12} is cross product coefficients. The optimal point of the regression equation was determined by Newton method using MATLAB version 14.

6. Treatment of the resulted hydrolyzate

The fermented SPB at the optimum conditions (after extraction) was subjected to autoclaving (121°C, 1.5atm, 20min) followed by sonication (5min in cycles of 0.5s/0.5s at 25°C). At each step the amount of the released reducing sugars in the supernatant after centrifugation for 10min at 150rpm and 4°C was determined.

7. Thin layer chromatography analysis

The clear supernatant after centrifugation was analyzed by TLC using propanol: water: ammonia (7: 2: 1 v/v) as the mobile phase (**Cabrera and Cutsem, 2005**) then the produced sugars were visualized using diphenyl amine-aniline spraying reagent (**Tanaka *et al*., 1999**).

8. Purification of the produced oligosaccharide

The resulted solution after filtration of the cell free supernatant using membrane filter (CA 0.2), was precipitated by cold absolute ethanol in a ratio 10:1 (ethanol:supernatant) and left overnight at 4°C to denaturant any excitant protein then it was centrifuged. The resulted precipitate was re-dissolved in distilled water and centrifuged (**Embaby *et al*., 2018**). Finally, the clear supernatant was air dried and stored at 4°C until further use.

9. Chemistry of the produced oligosaccharide

According to the spectrum obtained by Fourier Transform Infrared Spectroscopy (FTIR-8300, Shimadzu, Japan) for the dried COS, the functional groups and chemical bonds were determined.

10. Prebiotic activity

The ability of the purified COS to stimulate the growth of four probiotic strains obtained from the culture collection of the Department of Chemistry of Natural and Microbial products, National Research Center, Giza, Egypt, in compare to a pathogenic strain (*Escherichia coli* ATCC 8739) was examined as described by **Hussein *et al.*, (2015)**. MRS broth medium containing 0.2% of the produced COS was inoculated by the pre-cultured strains and incubated for 24h at 37°C then the absorbance was measured at 600nm. The prebiotic index was calculated as follow:

$$\text{Prebiotic index} = [A_{pro(24)} - A_{pro(0)}] / [A_{E(24)} - A_{E(0)}] \quad \text{Eq. (5)}$$

where $A_{pro(0)}$ and $A_{E(0)}$ are the initial absorbance of the probiotic and *Escherichia coli* respectively while $A_{pro(24)}$ and $A_{E(24)}$ are that after 24h.

11. Antioxidant activity

The antioxidant activity of the dried COS was determined according to its scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (**Brand-Williams *et al*., 1995**) in compare to chitin. The reaction was carried out by complete a volume of 3.9mL of DPPH radical (1.1 x 10^{-4}mol/L) in methanol solution to reach 4mL by adding 0.1mL of the sample solution (1%). After 30min in dark, the decrease in absorbance at 515nm was measured spectrophotometrically using Trolox as a standard. The results are expressed in µg Trolox Equivalents (TE)/mg of the dry sample.

12. Statistical analysis

All date reported in this study represents the average of the results obtained from three measurements of each experiment which originally performed in triplicates.

RESULTS

1. Oligosaccharide level

The bacterial strain *Bacillus cereus* SSW1 (under the previously mentioned fermentation condition described in the above section) produced total reducing sugar of 0.358mg N-acetyl glucosamine/mL.

2. Optimization of oligosaccharide production

2.1. Plackett-Burman design

The mean value of the released reducing sugars was represented in Table 1 in which a wide variation ranged from 0.286 to 1.095mg/mL was observed. The maximum amount of the released reducing sugars was achieved at run number 4 under the optimized conditions of fermentation period, 72hr; temperature, 37°C; period of microwave pretreatment, zero minute; K_2HPO_4, 1.5%; $MgSO_4$, 0.01%; KCl, 0.2% and $FeSO_4.7H_2O$, 0.01%.

Table 1. Plackett – Burman design.

Run number	Fermentation period (hour)	Temperature (°C)	Period of microwave pretreatment (minute)	K_2HPO_4 (%)	$MgSO_4$ (%)	KCl (%)	$FeSO_4.7H_2O$ (%)	Reducing sugar (mg/mL)
1	(24) -	(30) -	(0) -	(1.5) +	(0.1) +	(2) +	(0.01) -	0.315517
2	(72) +	(30) -	(0) -	(0.15) -	(0.01) -	(2) +	(0.1) +	0.66065
3	(24) -	(37) +	(0) -	(0.15) -	(0.1) +	(0.2) -	(0.1) +	0.286583
4	(72) +	(37) +	(0) -	(1.5) +	(0.01) -	(0.2) -	(0.01) -	1.09535
5	(24) -	(30) -	(1) +	(1.5) +	(0.01) -	(0.2) -	(0.1) +	0.381667
6	(72) +	(30) -	(1) +	(0.15) -	(0.1) +	(0.2) -	(0.01) -	0.96005
7	(24) -	(37) +	(1) +	(0.15) -	(0.01) -	(2) +	(0.01) -	0.295483
8	(72) +	(37) +	(1) +	(1.5) +	(0.1) +	(2) +	(0.1) +	0.79975

The multiple regression analysis of the data indicated that all of the tested variables significantly influencing the amount of the released COS (Table 2). The effect

of the seven independent variables was estimated by the coefficient values, the variable that exerted positive effect was maintained at a positive level while the one exerted negative effect was maintained at a negative level in the second phase of optimization. The analysis of variance (ANOVA) indicated that the model terms used in that study are highly significant, conducted from the high F value (2016.844) and the low Prob> F value ($2.39E^{-22}$).

Table 2. Multiple regression analysis of Plackett- Burman design.

Variables	**Coefficient**	***t*-statistics**	***P*-value**	**Confidence level (%)**
Intercept	-0.027			
Fermentation period (hour)	0.279569	109.3575	2E-24	100
Temperature (°C)	0.005689	7.788254	7.84E-07	100
Period of microwave pretreatment (minute)	0.019713	3.855418	0.001399	99.86
K_2HPO_4 (%)	0.072133	19.04565	2.03E-12	100
$MgSO_4$ (%)	-0.19792	-3.48381	0.003067	99.69
KCl (%)	-0.09059	-31.8922	6.54E-16	100
$FeSO_4.7H_2O$ (%)	-1.49375	-26.2936	1.36E-14	100
Model summary				
Multiple R	0.999434			
R^2	0.998868			
Adjusted R^2	0.998373			
Standard Error	0.012524			

The R^2 value of the selected model was 0.999 and the first order equation that described the correlation of the examined seven variables and the COS level could be presented as follows:

$$Y=-0.027 + 0.279569X_1 + 0.005689X_2 + 0.019713X_3 + 0.072133X_4 -0.19792X_5 - 0.09059X_6 - 1.49375X_7 \quad \text{Eq. (6)}$$

where Y is the released reducing sugars and X_1, X_2, X_3, X_4, X_5, X_6, X_7 are the fermentation period, temperature, period of microwave pretreatment, K_2HPO_4, $MgSO_4$, KCl and $FeSO_4.7H_2O$ respectively.

The calculated main effects of the examined variable were represented graphically in Fig. 1. The highest main effect was observed with the incubation period and the concentration of KCl, in which the former had positive sign and the latter had negative sign.

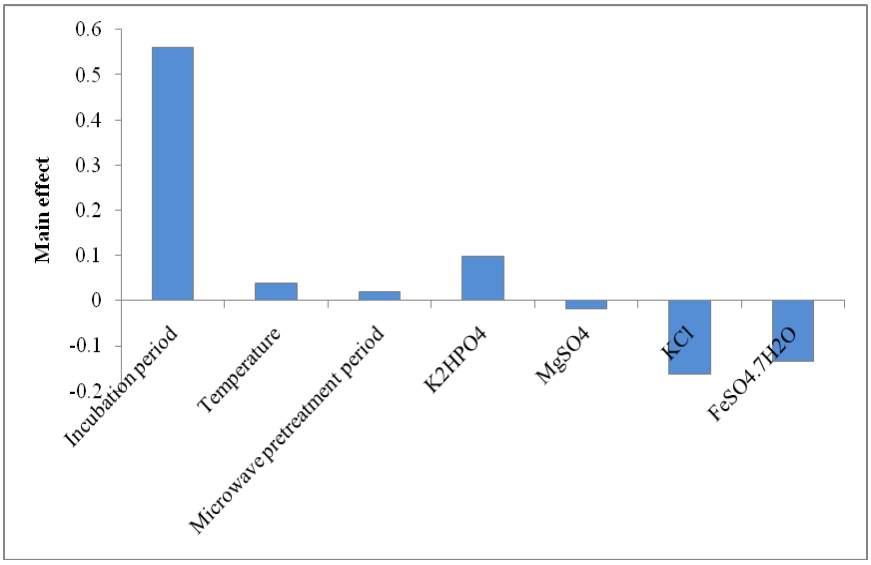

Fig. 1. The main effect of the examined variables on the production of COS.

2.2. Central composite design

The mean value of the experimental and the predicted reducing sugars was represented in Table 3. The optimum amount of the released reducing sugars (1.64mg/mL) was observed in run 3 from which the optimized level of the selected variables was conducted to be as follow: the fermentation period, 4days and KCl concentration, 0.1%.

Table 3. CCD for optimization of COS production.

Trial	Independent variable		Observed reducing sugar (mg/mL)	Predicted reducing sugar (mg/mL)	Residual
	X_1 Fermentation period (days)	X_2 KCl (%)			
1	2(-1)	0.1(-1)	0.768507	0.714357	0.05415
2	2(-1)	0.3(+1)	0.891961	0.93056	-0.0386
3	4(+1)	0.1(-1)	1.64125	1.420093	0.221157
4	4(+1)	0.3(+1)	1.128842	1.000436	0.128406
5	1(-∞)	0.2(0)	0.439505	0.401649	0.037856
6	5(+∞)	0.2(0)	1.048211	1.177261	-0.12905
7	3(0)	0(-∞)	1.125709	1.217689	-0.09198
8	3(0)	0.4(+∞)	1.014997	1.014235	0.000762
9	3(0)	0.2(0)	1.075784	1.080015	-0.00423
10	3(0)	0.2(0)	1.104193	1.080015	0.024178
11	3(0)	0.2(0)	1.000584	1.080015	-0.07943
12	3(0)	0.2(0)	0.999331	1.080015	-0.08068
13	3(0)	0.2(0)	1.037766	1.080015	-0.04225

The multiple regression analysis of the data was illustrated in Table 4 in which the R^2 value of the selected model was 0.86. The second order polynomial equation used to calculate the predicted values was:

$$Y = -0.97157 + 0.947673X_1 + 3.900844X_2 - 0.07264X_1^2 + 0.898678X_2^2 - 1.58965X_1X_2 \quad \text{Eq. (7)}$$

where Y is the released reducing sugars and X_1 and X_2 are the fermentation period and KCl concentration respectively. The three dimensional graph of the equation, plotted between the two independent variables and the released reducing sugar, was shown in Fig. 2.

Table 4. Analysis of CCD.

Term	Regression coefficient	Standard error	t- test	P-value
Intercept	-0.97157	0.223592	-4.3453	0.000125
X_1	0.947673	0.097233	9.746389	3.07E-11
X_2	3.900844	1.039115	3.754005	0.000672
X_1^2	-0.07264	0.012468	-5.8261	1.61E-06
X_2^2	0.898678	1.246754	0.720814	0.476099
X_1X_2	-1.58965	0.298399	-5.32728	7.02E-06
Model summary				
Multiple R	0.927731			
R^2	0.860686			
Adjusted R^2	0.839578			
Standard Error	0.103368			

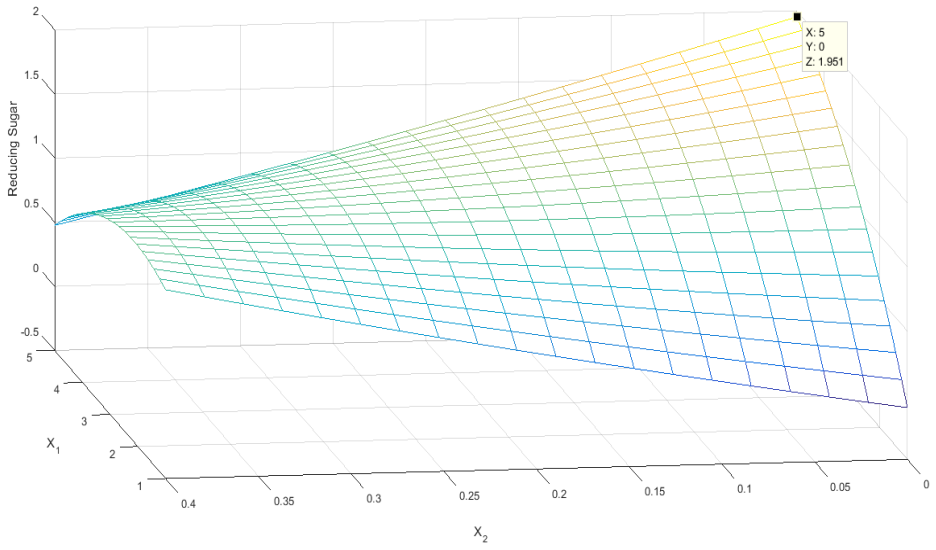

Fig. 2. Response surface plot showing the interactive effects of incubation period and KCl concentration on reducing sugar production.

The absolute average deviation (AAD) was calculated by equation 8 to be 6.77.

$$AAD = \{[\sum_{i=1}^{p}(|Yexp - Yprd|/Yexp)]/P\} \text{ X } 100 \quad \text{Eq. (8)}$$

where P, Yexp and Yprd are the number of the experiment, observed and predicted reducing sugar respectively.

The analysis of variance (ANOVA) for this model indicated that the study are statistically significant because the model terms had Prob> F value of 3.42E-13 (less than 0.05). Moreover, by plotting of the residuals (observed – predicted values) versus the observed response (Fig. 3), it was indicated that the residuals were spread throughout the experimental range.

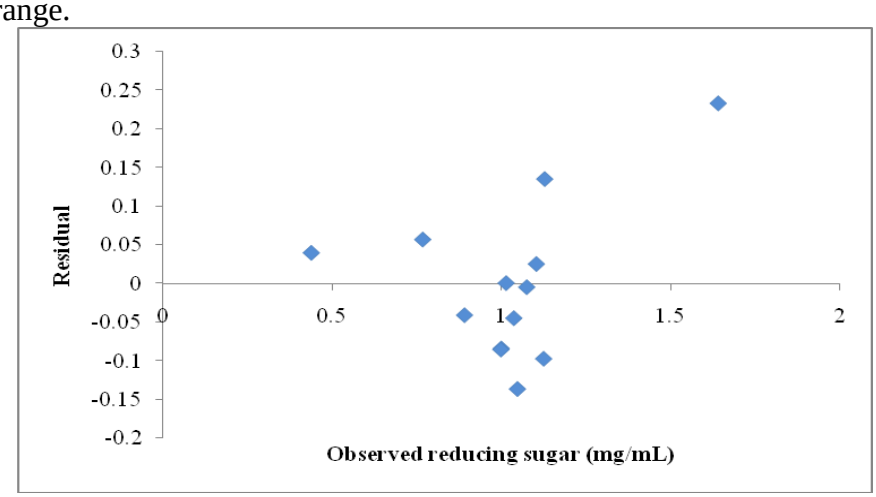

Fig. 3. Residual plot.

3. Treatment of the resulted hydrolyzate

The effect of additional treatments on the amount of the released reducing sugars produced under the optimized fermentation conditions was examined. The results indicated that autoclaving (121°C, 1.5atm, 20min) increased the released reducing sugars by 32% without the detection of any improvement by the sonication process (5min in cycles of 0.5s/0.5s at 25°C).

4. Thin layer chromatography analysis

The accumulated sugars in the resulted hydrolyzate were analyzed by TLC (Fig. 4), indicating the release of N-acetyl COS mixture.

Fig. 4. TLC plate of SPB hydrolyzate in which S1, S2, S3 are N-acetyl glucosamine, N-acetyl chitopentose and N-acetyl chitohexose standard respectively and A, B, C, D are the initial, after autoclaving, after filtration and ethanol precipitated hydrolyzates.

5. Fourier transform infrared analysis

The FTIR spectrum of the purified dried COS was shown in Fig. 5. The result indicated that the produced COS possessed the characteristic absorption bands of N-acetyl glucosamine, appeared at 3430cm^{-1} (OH and NH stretching), 1640cm^{-1}(amide I) and 1550cm^{-1} (amide 2). Also the result indicated the similarity of the FTIR spectrum of the produced COS to that of the parent chitin.

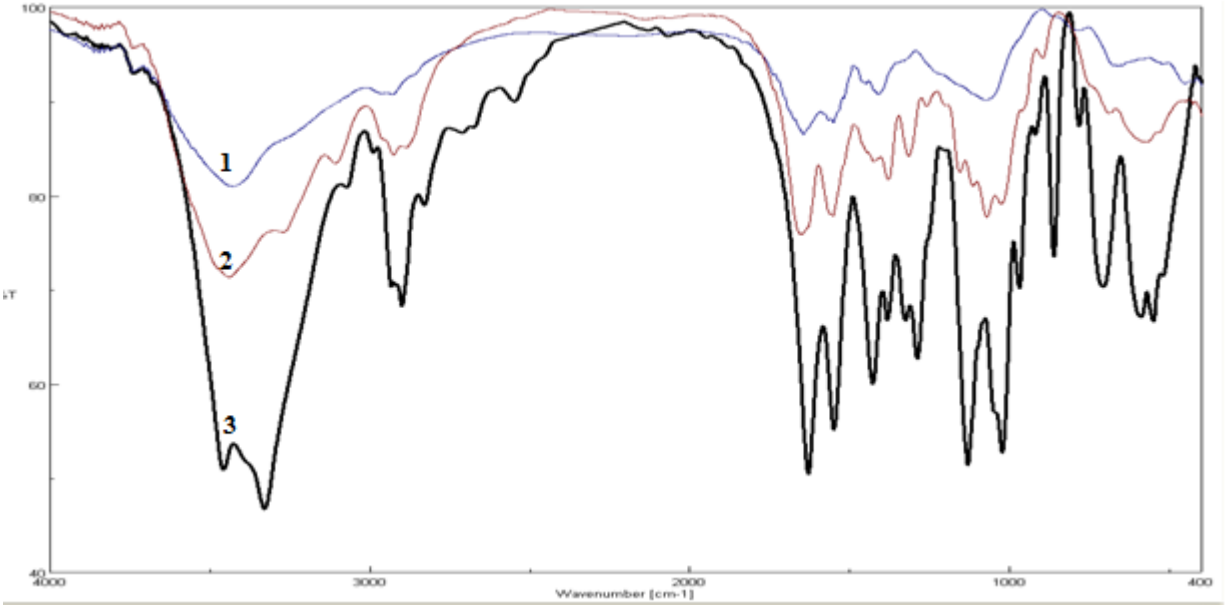

Fig. 5. FTIR spectrum of (1) COS, (2) chitin and (3) N-acetyl glucosamine.

6. Prebiotic activity

The prebiotic activity of the purified COS was evaluated using four probiotics and the results shown in Table 5. The results indicated that the prebiotic index was higher than one for all of the tested strains. Moreover, the highest prebiotic index (3.23) was observed for *Lactobacillus plantarum.*

Table 5. Prebiotic activity of chitin-oligosaccharide.

Probiotic strain	Prebiotic index
Lactobacillus lactis	1.542446
Lactobacillus plantarum	3.231655
Lactobacillus acidophilus	1.292086
Pediococcusacidilactici	1.774101

7. Antioxidant activity

The scavenging activity of the purified dried COS for DPPH radical was examined. The results indicated that the total dried COS possessed antioxidant activity of 205.89±10.97µg TE/mg without detection of any antioxidant activity for chitin.

DISCUSSION

Chitin is the main constituent of the crustacean shell (**Yan and Chen, 2015**). Typically, the use of chitin as a carbon source for the cultivation of microorganisms would result in the production of two concomitant extracellular end products; chitinolytic

enzymes as the main product and chitin hydrolysis products as co-products. Chitin hydrolysis products particularly COS have attracted a growing interest as they possess various biological activities. The bacterial strain *Bacillus cereus* SSW1 was previously reported to be capable of producing COS by the fermentation of SPB as a sole carbon and nitrogen source (**Ismail, 2019**). Since the overall cost of any bioprocess is a major obstacle in its industrial application and the growth medium is estimated to cost around 40% of the overall production cost (**Okoroma *et al*., 2012**)**.** So, the adjustment of a low cost production medium that works effectively and efficiently for massive production of the aimed end product is one of the most important detected challenges in upstream processing. In the current study, statistical optimization of COS production was performed by applying response surface methodology that has been reported to be a promising tool in the optimization of various microbiological and biotechnological processes (**Yolmeh & Jafari, 2017 and Ismail *et al*., 2019c)**. The optimization was achieved by applying two phase model. Initially, seven variables were examined for their influence on the level of the production of COS by applying PBD. The R^2 value of the applied model was 0.999, indicating its high accuracy as it prescribed that 99.9% of the variation was attributed to the independent variables. **Joglekar and May, (1987)** reported that the fitness and the accuracy of the applied model were indicating when the R^2 value was more than 80%. The analysis of the results indicated that all of the examined variables were significant. Additionally, the calculated main effects indicated that the fermentation period, temperature, period of microwave pretreatment and K_2HPO_4 concentration had positive values while the concentration of $MgSO_4$, KCl and $FeSO_4.7H_2O$ had negative values. The positive values indicates that the tested variable exerts more effect as it adjusted at the high level while the negative values indicates that the tested variable exerts more effect when adjusted at the low level. The incubation period and the concentration of KCl were the variables that exerted the highest main effects so they were selected for further optimization. The second phase of optimization was achieved by applying CCD in which the R^2 and AAD values were 0.86 and 6.77 respectively. **Ghorbannezhad *et al*., (2016) and Yolmeh and Jafari, (2017)** reported that the convenient values of R^2 and AAD, expressed the correct behavior of the applied model as well as the efficiency of the applied model in the optimization process. Moreover the model is in average correct for all of the observed results as it is cleared from the residual analysis. The optimized conditions was conducted to be, 5g of microwave pretreated SPB moistened with 10mL of moistening agent composed of K_2HPO_4, 1.5%; $MgSO_4$, 0.01%; KCl, 0.1%; $FeSO_4.7H_2O$, 0.01% and incubated for 4days at 37°C. The use of microwave heating has been reported to be capable for the homogeneously transfer of heat at the molecular level within the sample matrix (**Prajapat and Gogate, 2015**) and recently, it has been utilized to assist either the chemical or the enzymatic hydrolysis of SPB as it can shorten the reaction time and increase the product yield (**Xiao *et al*., 2019 and Zhao *et al*., 2019**). The statistical optimization of the bacterial hydrolysis of SPB for the production of COS was studied by **Halder *et al*., (2013)** at which the optimum productivity was 5.5mg/g, achieved after 66.4h fermentation at 37.6°C using *Aeromonas hydrophila* SBK1, which was lower than 16.4mg/g reported in the current study.

The effect of additional pre-treatment on the amount of the released reducing sugars under the optimized fermentation conditions was examined. An increase by 32%

was indicated by autoclaving, combining the advantage of sterilization with the increase in the amount of the released reducing sugars. The maximum amount of reducing sugars released in the fermentation broth was increased to reach 21.7mg/g of SPB with hydrolysis percentage of 58.6%. In subsequent step, the released sugars were analyzed by TLC and FTIR confirming the release of oligomers of N-acetyl glucosamine with the indication of the release of chitopentose and chitohexose oligomers. The DP of the produced COS in the current study was higher than that reported by **Halder *et al*., (2013)** that indicated the release of reducing sugars composed of 57.5% N-acetyl glucosamine and 39.2% chitobiose by the bacterial hydrolysis of SPB.

Additionally, the total population growth of four probiotics using the produced COS was estimated in compare to *Escherichia coli.* The results indicated that the produced COS capable for the proliferation of the growth of all the examined probiotics with a prebiotic index range from 1.29 to 3.23. Similar result was reported by **Selenius *et al*., (2018)** that indicated the promoting effect of COS to the growth of *lactobacillus rhamnosus* GG. Moreover, the antioxidant activity of the purified COS was examined by determining its scavenging activity of DPPH radical. The result indicated that the produced COS possessed antioxidant activity of 205.89µg TE/mg without the detection of any antioxidant activity to its parent compound (chitin). This result was higher than the previous antioxidant activity (55.89µg TE/ mg) reported for the low molecular weight COS extracted from TLC **(Ismail, 2019)** that may attributed to the increase in the amino and the hydroxyl groups. The antioxidant activity of COS has been reported to be directly correlated to these groups that react with the unstable free radicals forming a stable macromolecules (**Wang *et al*., 2011 ; Avelelas *et al*., 2019**).

CONCLUSION

Direct production of chitin-oligosaccharides from shrimp byproducts was achieved by its bacterial fermentation using *Bacillus cereus* strain SSW1. Statistical optimization of the production was achieved by applying Plackett-Burman design followed by central composite design. The optimum productivity was further increased by 32% after autoclaving that consequently indicated that the achieved hydrolysis percentage was 58.6%. Finally, the produced mixture of chitin-oligosacharides could be suggested as a suitable candidate in the production of functional food as it possessed prebiotic and antioxidant activity.

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